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PATENT -- NO FEE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In Re: Application of
PETER NASH ET AL

Serial No.: 10/038,260

Filed: January 7, 2002

For: IMMUNOGEN ADHERENCE INHIBITOR
AND METHOD OF MAKING AND
USING SAME

Case Docket No.: C150.12.3C

Group Art Unit 1644

Exr. P. Huynh

LETTER

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22131-1450

Sir:

Enclosed are the original and three (3) copies of Appellants' Reply to Examiner's Answer
under 37 CFR 1.193.

Respectfully submitted,

PETER NASH ET AL

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I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in
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FEBRUARY 14, 2005
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Richard John Bartz
Name of applicant, assignee, or Registered Rep.

Richard John Bartz
Signature

FEBRUARY 14, 2005
Date of Signature



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APPELLANTS' REPLY TO EXAMINER'S ANSWER 37 CFR 1.193

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22131-1450

Sir:

This Reply is in response to the Examiner's Answer of December 17, 2004.

1. RELATED APPEALS AND INTERFERENCES

There are three (3) related appeals pending before the Board of Patent Appeals and Interferences which directly affect or are directly affected by or have a bearing on this application. The related patent applications are: U.S. Application Serial No. 09/616,843 (parent application); Serial No. 10/025,567; and Serial No. 10/039,977.

2. STATUS OF CLAIMS

Claims 1, 3 and 5 to 38 are pending in this application. No claims have been allowed.

3. STATUS OF AMENDMENTS FILED SUBSEQUENT TO FINAL REJECTION

An Amendment 37 CFR 1.116 filed February 2, 2005 corrects informal language in

Claims 32, 34 and 36. Appellants have not received a response to this amendment.

4. CLAIMS

Claims 1, 3 and 5 to 38, with amendments to Claims 32, 34 and 36, are presented in attached Appendix A.

5. GROUPING OF CLAIMS

The Examiner on page 4 of the Examiner's Answer does not agree with Appellants that the claims fall into three groups and that the claims of the three groups do not stand and fall together.

Group II Claims 9-10,12-13, 15-16, 18-19, 21-22, 24-25 and 27-28 include the drying of the entire contents of the eggs with yolk IgY and albumin IgM and IgA immunoglobulins and then coating the dried egg contents on a dry feed carrier. Group III Claims 6-7 and 29-38 do not include the method of drying the egg contents and then coating the dried egg contents on a dry feed carrier. The wet egg contents are coated on a dry feed carrier. The dry feed carrier absorbs moisture from the egg contents to allow the microbial adherence inhibitor to be stored and transported to a user location.

Group I Claims 1, 3, 5, 8, 17, 20, 23 and 26 do not include the dry feed carrier that is coated by the egg contents included in Group II and III claims.

The use of the dry feed carrier in Groups II and III claims is relevant, novel and patentable in view of the prior art. The egg content drying process is included in the claimed method for the production of the microbial adherence inhibitor.

6. 35 USC § 112 NEW MATTER REJECTION OF CLAIMS 5 AND 32 TO 38

The Examiner on page 9, lines 16 to 23 of the Examiner's Answer outlines the rejection of Claims 5 and 32 to 38 under 35 USC § 112 as containing new matter. The alleged new matter is identified by the terms "living being" as used in these claims.

The Amendment 37 CFR 1.116 to Claims 32, 34 and 36 has changed the term "living being" to --animals-- to correct informal language in these claims. The term animals has an antecedent in Claims 32, 34 and 36. These amendments overcome the 35 USC § 112 new matter rejection.

Claims 5 and 38 define a method for the production of a microbial adherence inhibitor for administration to a living being to inhibit the adherence of a colony-forming immunogen in the digestive tract of the living being. The colony-forming immunogen is from the class consisting of *E. coli*, *Listeria*, *Salmonella* and *Campylobacter*. The Examiner states that the terms "living being" departs from the specification and claims as originally filed. The terms "living being" has not been considered indefinite by the Examiner. The specification describes the method for the production of a microbial adherence inhibitor for administration to animals and hosts. The term hosts is included in the specification in paragraph 0002, lines 7-10; paragraph 0027, lines 1-2; and paragraph 0066, lines 1-2. A host, broadly, is an organism that contains another organism. More specifically, a host is a living animal including a humanoid that affords lodgment or subsistence to a commensal organism. The word "being" is a host that has existence or lives. The common terms for a living host is a living being. The terms "living being" as included in Claims 5 and 38 are not indefinite and new matter to this application.

7. CLAIM REJECTIONS -- 35 USC § 103

The primary references in the rejections under 35 USC § 103 of Claims 1, 3 and 5 to 38 are U.S. Patent No. 5,080,895 (*Tokoro et al*) and *Yokoyama et al*.

Tokoro et al and *Yokoyama et al* do not disclose or teach a method for the production of a microbial adherence inhibitor for administration to a living being including animals to inhibit adherence of a colony-forming immunogen in the rumen or intestinal tracts of animals by binding IgY immunoglobulins to a protein-wasting immunogen with the assistance of IgM and IgA

immunoglobulins. IgM and IgA are multivalent and strong binding antibodies. The colony-forming immunogen is from the class consisting of *E. coli*, *Listeria*, *Salmonella* and *Campylobacter*.

Tokoro '895 discloses a method of inhibiting diarrhea in animals with bird antibody IgY using the yolks, albumin and the yolks of eggs. This method is related to the use of raw eggs by cattle herders to treat scours (diarrhea in cattle caused by intestinal infection). *Tokoro '895* is directed to a specific antibody containing substance from eggs and method of production and use thereof for the prevention and treatment of colibacillosis and diarrhea in animals. There is no disclosure in *Tokoro '895* of an IgY immunoglobulin that binds to colony-forming illness-causing immunogens with the assistance of IgM and IgA immunoglobulins. The antibody containing substance also is used as a nutrition supplement.

Tokoro '895 does not coat a dry feed carrier with a mixed egg yolk and albumin product.

The object of the *Tokoro '895* disclosure is to administer to animals affected by an intestinal infection disease for therapeutic purposes. *Column 4, lines 1-4*. The *Tokoro '895* substance is also useful in the treatment of various infectious diseases, additives in food for livestock, cosmetics and medicines. *Column 4, lines 16-21*. Appellants' claimed method is not a treatment of a disease in animals. Appellants' method is the prevention of illnesses in humans by eliminating the illness-causing immunogens in animal meat. Appellants have discovered a new and useful method of preventing, as opposed to treating, illnesses in humans caused by a colony-forming illness-causing immunogen from the class consisting of *E. coli*, *Listeria*, *Salmonella* and *Campylobacter*.

The Examiner has acknowledged that the teachings of *Tokoro '895* do not include "the method wherein the antibody in the eggs including IgY immunoglobulins in the yolks of the eggs whereby the IgY immunoglobulins bind to a targeted colony-forming illness-causing

immunogen, said binding being assisted by the IgM and IgA immunoglobulins to inhibit adherence of the targeted-colony forming illness-causing immunogen in the intestinal tract of the animals." *Office Action of July 28, 2004, page 7, lines 9-14 of related Application Serial No. 10/039,977.*

The Examiner has outlined the teaching of *Yokoyama et al* on page 10, lines 21-31; page 19, lines 19-29; page 31, lines 25-32; page 32, lines 1-3; page 38, lines 17-20; page 40, lines 18-30 and page 55, lines 7-17.

"Yokoyama *et al* teach a method of producing a microbial adherence inhibitor such as IgY for administration to food animal such as piglets to inhibit the adherence of a targeted colony forming immunogen such as *E coli* in the intestinal tracts of the reference animal (See abstract, in particular). The reference method comprises inoculating the hen in or about to reach their egg laying age with the reference colony forming immunogen such as *E coli* (See page 999, Immunization with fimbrial vaccine, in particular), harvesting the eggs lay be the chickens and separating the IgY from the yolk (See page 999, column 2, Separation of antibodies from chicken egg yolk, in particular), drying the separated IgY (See page 1000, column 2, Production of antibody powders by spray drying, in particular) and when administering yolk antibodies to the living being such as neonatal piglets, the yolk antibodies inhibit the adherence of the colony forming immunogen *E coli* in the digestive tract."

The Examiner's analysis of this *Yokoyama et al* publication does not agree with the disclosure of this publication. A copy of *Yokoyama et al*, Vaccine 16(4): 388-393, February 1998, is attached Appendix B. The Examiner refers to pages 999, column 2, and page 1000, column 2. The *Yokoyama et al* publication only has pages 388 to 393. The *Yokoyama et al* publication relates to the efficacy of chicken egg yoke antibodies for controlling experimental salmonellosis in mice. There is no reference to piglets. Only egg yolk immunoglobulin IgG was used in the experiment. *Page 389, last paragraph.* The *Yokoyama et al* publication discloses isolation of antibodies from chicken egg yolk. Immunoglobulin G (IgG) egg yolk was diluted with distilled water and mixed with ethyl alcohol. The mixture was centrifuged. The supernatant which contained the IgG was purified. This process does not disclose or suggest

Appellants' method for production of a microbial adherence inhibitor defined in Claims 1, 3, 5, 8, 11, 14 and 17. There is no disclosure in *Yokoyama et al* of IgY, IgM and IgA immunoglobulins with IgY immunoglobulins binding to protein-wasting immunogens to inhibit the ability of protein-wasting immunogens to adhere to the intestinal tracts of animals and the binding process being assisted or helped by IgM and IgA immunoglobulins.

The teachings of the secondary references, *Pimental '489*, *Stolle '018*, *Adalsteinsson '878*, *Kuby et al*, *Abaza et al*, *Stryer et al*, *Kaspers et al*, *Trinchieri et al* and *Sugita-Konishi et al* are described in Appellants' Brief, pages 15-17. The separate and combined teachings of the secondary references do not provide any motivating facts or suggestions to a person skilled in the art to produce the claimed method. There is no teaching in the secondary references of a method of producing a microbial adherence inhibitor that binds IgY immunoglobulins combined with IgM and IgA immunoglobulins to protein-wasting immunogens to inhibit the ability of the protein-wasting immunogens to adhere to the rumen or intestinal tracts of food animals and to reduce the ability of the immunogens to multiply in the rumen or intestinal tracts.

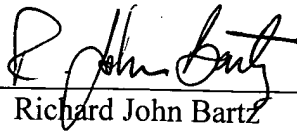
In summary, Appellants submit that there is no clear and particular objective evidence of record showing motivation to one skilled in the art to combine the myriad of references. The teachings of the primary references, *Tokoro et al* and *Yokoyama et al*, and the multitude of secondary references do not suggest to a person skilled in the art Appellants' claimed method for the production of a microbial adherence inhibitor for administration to animals to inhibit adherence of a colony-forming immunogen in the rumen or intestinal tracts of the animals by binding IgY immunoglobulins to a colony-forming immunogen with the assistance of IgM and IgA immunoglobulins.

The reversal of the rejections as to Claims 1, 3 and 5 to 38 is requested.

Respectfully submitted,

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APPENDIX A

1. A method for the production of a microbial adherence inhibitor for administration to food animals to inhibit the adherence of a targeted colony-forming immunogen in the rumen or intestinal tracts of said food animals, which method comprises:
 - A. Inoculating female chickens, in or about to reach their egg laying age, with a particular targeted colony-forming immunogen;
 - B. Allowing a period of time sufficient to permit the production in the eggs of the chickens of antibody to the target colony-forming immunogen, said antibody in the eggs including IgY immunoglobulins in the yolks of the eggs and IgM and IgA immunoglobulins in the albumin of the eggs;
 - C. Harvesting the eggs laid by the chickens;
 - D. Separating the entire contents of said harvested eggs from the shells; and
 - E. Drying said separated entire contents of said eggs, said dried entire contents of said eggs when administered to food animals with animal feed promoting the growth of the food animals by decreasing the waste of dietary protein caused by the presence of protein-wasting immunogen in the rumen or intestinal tracts of the food animals by binding the IgY immunoglobulins to the protein-wasting immunogen being assisted by the IgM and IgA immunoglobulins to inhibit the ability of the targeted colony-forming immunogen to adhere to the rumen or intestinal tracts of the animals.
3. The method according to Claim 1 wherein: said targeted colony-forming immunogen is from the class consisting of *P. anaerobius*, *C. sticklandii* and *C. aminophilum*.
5. A method for the production of a microbial adherence inhibitor for administration to a living being to inhibit the adherence of a colony forming immunogen in the digestive tract of

the living being, said colony-forming immunogen is from the class consisting of *E. coli*, *Listeria*, *Salmonella* and *Campylobacter*, which method comprises:

- A. Inoculating female chickens in or about to reach their egg laying age with the colony-forming immunogen;
- B. Allowing a period of time sufficient to permit the production in the eggs of the chickens of antibody to the colony-forming immunogen, said antibody in the eggs including IgY immunoglobulins in the yolks of the eggs and IgM and IgA immunoglobulins in the albumin of the eggs;
- C. Harvesting the eggs laid by the chickens;
- D. Separating the entire contents of said harvested eggs from the egg shells; and
- E. Drying said separated entire contents of said eggs, said dried entire contents of said eggs when administered to the living being inhibiting the adherence of the colony-forming immunogen in the digestive tract by binding the IgY immunoglobulins to the colony-forming immunogen, said binding of the IgY immunoglobulins to the colony-forming immunogen being assisted by the IgM and IgA immunoglobulins.

6. A method for the production of a microbial adherence inhibitor for administration to food animals to inhibit the adherence of a targeted colony-forming immunogen in the rumen or intestinal tracts of said food animals produced by the method of:

- A. Inoculating female chickens, in or about to reach their egg laying age, with a particular target colony-forming immunogen;
- B. Allowing a period of time sufficient to permit the production in the eggs of the chickens of antibody to the target colony-forming immunogen, said antibody in the eggs

including IgY immunoglobulins in the yolks of the eggs and IgM and IgA immunoglobulins in the albumin of the eggs;

- C. Harvesting the eggs laid by the chickens;
- D. Separating the entire contents of said harvested eggs from the egg shells;
- E. Providing a dry feed carrier material; and
- F. Coating said dry feed carrier material with the separated entire contents of said harvested eggs, said dry food carrier material coated with the entire contents of said eggs when administered to the living being inhibiting the adherence of colony-forming immunogen in the digestive tract by binding the IgY immunoglobulins to the colony-forming immunogen, said binding of the IgY immunoglobulins to the colony-forming immunogen being assisted by the IgM and IgA immunoglobulins.

7. The method of Claim 6 wherein: providing a dry feed carrier material from a group of materials including soybean hulls, rice hulls, corn, cottonseed hulls, distilled dried grains and beet pulp.

8. A method for the production of a microbial adherence inhibitor for administration to food animals to inhibit the adherence of a colony-forming immunogen in the rumen or intestinal tracts of said food animals, said immunogen is P antigen from *P. anaerobius*, which method comprises:

- A. Inoculating female birds, in or about to reach their egg laying age, with P antigen from *P. anaerobius*;

- B. Allowing a period of time to permit the production in the birds and eggs laid by the birds of antibody to P antigen from *P. anaerobius*, said antibody in the eggs including

IgY immunoglobulins in the yolks of the eggs and IgM and IgA immunoglobulins in the albumin of the eggs;

C. Harvesting the eggs laid by the birds;

D. Separating the entire contents of said harvested eggs from the egg shells;

and

E. Drying said entire contents of said separated eggs, said dried entire contents of said eggs when administered to food animals with animal feed promoting the growth of the food animals by decreasing the waste of dietary protein caused by the presence of a protein-wasting immunogen in the rumen of intestinal tracts of the food animals by binding the IgY immunoglobulins to the protein-wasting immunogen, said binding of the IgY immunoglobulins to the protein-wasting immunogen being assisted by the IgM and IgA immunoglobulins to inhibit the ability of the protein-wasting immunogen to adhere to the rumen or intestinal tracts of the animals.

9. The method of Claim 8 including: providing a dry carrier material, said drying of the separated entire contents of said eggs is achieved by coating the dry carrier material with the separated entire contents of said eggs.

10. The method of Claim 9 wherein: the dry carrier material is from a group of materials including soybean hulls, rice hulls, corn, cottonseed hulls, distilled dried grains and beet pulp.

11. A method for the production of a microbial adherence inhibitor for administration to food animals to inhibit the adherence of a colony-forming immunogen in the rumen or intestinal tracts of said food animals, said immunogen is CS antigen from *C. sticklandii*, said method comprising:

A. Inoculating female birds, in or about to reach their egg laying age, with CS antigen from *C. sticklandii*;

B. Allowing a period of time to permit the production in the birds and eggs laid by the birds of antibody to CS antigen from *C. sticklandii*, said antibody in the eggs including IgY immunoglobulins in the yolks of the eggs and IgM and IgA immunoglobulins in the albumin of the eggs;

C. Harvesting the eggs laid by the birds;

D. Separating the entire contents of said harvested eggs from the egg shells;
and

E. Drying said entire contents of said separated eggs, said dried entire contents of said eggs when administered to food animals with animal feed promoting the growth of the food animals by decreasing the waste of dietary protein caused by the presence of a protein-wasting immunogen in the rumen or intestinal tracts of the food animals by binding the IgY immunoglobulins to the protein-wasting immunogen, said binding of the IgY immunoglobulins to the protein-wasting immunogen being assisted by the IgM and IgA immunoglobulins to inhibit the ability of the protein-wasting immunogen to adhere to the rumen or intestinal tracts of the animals.

12. The method of Claim 11 including: providing a dry carrier material, said drying of the separated entire contents of said eggs is achieved by coating the dry carrier material with the separated entire contents of said eggs.

13. The method of Claim 12 wherein: the dry carrier material is from a group of materials including soybean hulls, rice hulls, corn, cottonseed hulls, distilled dried grains and beet pulp.

14. A method for the production of a microbial adherence inhibitor for administration to food animals to inhibit the adherence of a colony-forming immunogen in the rumen or intestinal tracts of said food animals, said immunogen is CA antigen from *C. aminophilum*, said method comprising:

A. Inoculating female birds, in or about to reach their egg laying age, with CA antigen from *C. aminophilum*;

B. Allowing a period of time to permit the production in the birds and eggs laid by the birds of antibody to CA antigen from *C. aminophilum*, said antibody in the eggs including IgY immunoglobulins in the yolks of the eggs and IgM and IgA immunoglobulins in the albumin of the eggs;

C. Harvesting the eggs laid by the birds;

D. Separating the entire contents of said harvested eggs from the egg shells;
and

E. Drying said entire contents of said separated eggs, said dried entire contents of said eggs when administered to food animals with animal feed promoting the growth of the food animals by decreasing the waste of dietary protein caused by the presence of a protein-wasting immunogen in the rumen or intestinal tracts of the food animals by binding the IgY immunoglobulins to the protein-wasting immunogen, said binding of the IgY immunoglobulins to the protein-wasting immunogen being assisted by the IgM and IgA immunoglobulins to inhibit the ability of the protein-wasting immunogen to adhere to the rumen or intestinal tracts of the animals.

15. The method of Claim 14 including: providing a dry carrier material, said drying of the separated entire contents of said eggs is achieved by coating the dry carrier material with the separated entire contents of said eggs.

16. The method of Claim 15 wherein: the dry carrier material is from a group of materials including soybean hulls, rice hulls, corn, cottonseed hulls, distilled dried grains and beet pulp.

17. A method for the production of a microbial adherence inhibitor for administration to food animals to inhibit the adherence of a colony-forming immunogen in the rumen or intestinal tracts of said food animals, said immunogen is *E. coli* antigen from *E. coli*, said method comprising:

A. Inoculating female birds, in or about to reach their egg laying age, with the *E. coli* colony-forming immunogen;

B. Allowing a period of time to permit the production in the birds of antibody to the *E. coli* immunogen, said antibody in the eggs including IgY immunoglobulins in the yolks of the eggs and IgM and IgA immunoglobulins in the albumin of the eggs;

C. Harvesting the eggs laid by the birds;

D. Separating the entire contents of said harvested eggs from the egg shells;
and

E. Drying said separated entire contents of said separated eggs, said dried entire contents of said eggs when administered to food animals with animal feed promoting the growth of the food animals by decreasing the waste of dietary protein caused by the presence of a protein-wasting immunogen in the rumen or intestinal tracts of the food animals by binding the IgY immunoglobulins to the protein-wasting immunogen, said binding of the IgY

immunoglobulins to the protein-wasting immunogen being assisted by the IgM and IgA immunoglobulins to inhibit the ability of the protein-wasting immunogen to adhere to the rumen or intestinal tracts of the animals.

18. The method of Claim 17 including: providing a dry carrier material, said drying of the separated entire contents of said eggs is achieved by coating the dry carrier material with the separated entire contents of said eggs.

19. The method of Claim 18 wherein: the dry carrier material is from a group of materials including soybean hulls, rice hulls, corn, cottonseed hulls, distilled dried grains and beet pulp.

20. A method for the production of a microbial adherence inhibitor for administration to food animals to inhibit the adherence of a colony-forming immunogen in the rumen or intestinal tracts of said food animals, said immunogen is *Listeria* antigen from *Listeria*, said method comprising:

A. Inoculating female birds, in or about to reach their egg laying age, with the *Listeria* colony-forming immunogen;

B. Allowing a period of time to permit the production in the birds of antibody to the *Listeria* immunogen, said antibody in the eggs including IgY immunoglobulins in the yolks of the eggs and IgM and IgA immunoglobulins in the albumin of the eggs;

C. Harvesting the eggs laid by the birds;

D. Separating the entire contents of said harvested eggs from the egg shells;

and

E. Drying the entire contents of said separated eggs, said dried entire contents of said eggs when administered to food animals with animal feed promoting the growth of the

food animals by decreasing the waste of dietary protein caused by the presence of a protein-wasting immunogen in the rumen or intestinal tracts of the food animals by binding the IgY immunoglobulins to the protein-wasting immunogen, said binding of the IgY immunoglobulins to the protein-wasting immunogen being assisted by the IgM and IgA immunoglobulins to inhibit the ability of the protein-wasting immunogen to adhere to the rumen or intestinal tracts of the animals.

21. The method of Claim 20 including: providing a dry carrier material, said drying of the separated entire contents of said eggs is achieved by coating the dry carrier material with the separated entire contents of said eggs.

22. The method of Claim 21 wherein: the dry carrier material is from a group of materials including soybean hulls, rice hulls, corn, cottonseed hulls, distilled dried grains and beet pulp.

23. A method for the production of a microbial adherence inhibitor for administration to food animals to inhibit the adherence of a colony-forming immunogen in the rumen or intestinal tracts of said food animals, said immunogen is *Salmonella* antigen from *Salmonella*, said method comprising:

A. Inoculating female birds, in or about to reach their egg laying age, with the *Salmonella* colony-forming immunogen;

B. Allowing a period of time to permit the production in the birds of antibody to the *Salmonella* immunogen, said antibody in the eggs including IgY immunoglobulins in the yolks of the eggs and IgM and IgA immunoglobulins in the albumin of the eggs;

C. Harvesting the eggs laid by the birds;

D. Separating the entire contents of said harvested eggs from the egg shells;
and

E. Drying the entire contents of said separated eggs, said dried entire contents of said eggs when administered to food animals with animal feed promoting the growth of the food animals by decreasing the waste of dietary protein caused by the presence of a protein-wasting immunogen in the rumen or intestinal tracts of the food animals by binding the IgY immunoglobulins to the protein-wasting immunogen, said binding of the IgY immunoglobulins to the protein-wasting immunogen being assisted by the IgM and IgA immunoglobulins to inhibit the ability of the protein-wasting immunogen to adhere to the rumen or intestinal tracts of the animals.

24. The method of Claim 23 including: providing a dry carrier material, said drying of the separated entire contents of said eggs is achieved by coating the dry carrier material with the separated entire contents of said eggs.

25. The method of Claim 24 wherein: the dry carrier material is from a group of materials including soybean hulls, rice hulls, corn, cottonseed hulls, distilled dried grains and beet pulp.

26. A method for the production of a microbial adherence inhibitor for administration to food animals to inhibit the adherence of a colony-forming immunogen in the rumen or intestinal tracts of said food animals, said immunogen is *Campylobacter* antigen from *Campylobacter*, said method comprising:

A. Inoculating female birds, in or about to reach their egg laying age, with the *Campylobacter* colony-forming immunogen;

B. Allowing a period of time to permit the production in the birds and eggs of antibody to the *Campylobacter* immunogen, said antibody in the eggs including IgY immunoglobulins in the yolks of the eggs and IgM and IgA immunoglobulins in the albumin of the eggs;

C. Harvesting the eggs laid by the birds;

D. Separating the entire contents of said harvested eggs from the egg shells;
and

E. Drying the entire contents of said separated eggs, said dried entire contents of said eggs when administered to food animals with animal feed promoting the growth of the food animals by decreasing the waste of dietary protein caused by the presence of a protein-wasting immunogen in the rumen or intestinal tracts of the food animals by binding the IgY immunoglobulins to the protein-wasting immunogen, said binding of the IgY immunoglobulins to the protein-wasting immunogen being assisted by the IgM and IgA immunoglobulins to inhibit the ability of the protein-wasting immunogen to adhere to the rumen or intestinal tracts of the animals.

27. The method of Claim 26 including: providing a dry carrier material, said drying of the separated entire contents of said eggs is achieved by coating the dry carrier material with the separated entire contents of said eggs.

28. The method of Claim 27 wherein: the dry carrier material is from a group of materials including soybean hulls, rice hulls, corn, cottonseed hulls, distilled dried grains and beet pulp.

29. A method for the production of a microbial adherence inhibitor for administration to food animals to inhibit the adherence of a targeted colony-forming immunogen in the rumen or intestinal tracts of said food animals, which method comprises:

A. Inoculating female birds, in or about to reach their egg laying age, with the particular targeted colony-forming immunogen;

B. Allowing a period of time sufficient to permit the production in the bird of antibody to the targeted immunogen, said antibody in the eggs including IgY immunoglobulins in the yolks in the eggs and IgM and IgA immunoglobulins in the albumin of the eggs;

C. Harvesting the eggs laid by the birds;

D. Separating the entire contents of said eggs from the egg shells;

E. Providing a dry carrier material; and

F. Coating said dry carrier material with the entire contents of said eggs, said dried entire contents of said eggs when administered to food animals with animal feed promoting the growth of the food animals by decreasing the waste of dietary protein caused by the presence of a protein-wasting immunogen in the rumen or intestinal tracts of the food animals by binding the IgY immunoglobulins to the protein-wasting immunogen, said binding of the IgY immunoglobulins to the protein-wasting immunogen being assisted by the IgM and IgA immunoglobulins to inhibit the ability of the protein-wasting immunogen to adhere to the rumen or intestinal tracts of the animals.

30. The method of Claim 29 wherein: the dry carrier material is from a group of materials including soybean hulls, rice hulls, corn, cottonseed hulls, distilled dried grains and beet pulp.

31. The method of Claim 29 wherein: said target-forming immunogen is from the class consisting of *P. anaerobius*, *C. sticklandii* and *C. aminophilium*.

32. A method for the production of a microbial adherence inhibitor for administration to food animals to inhibit the ability of the immunogen to adhere to the rumen or intestinal tracts of food animals to reduce the ability of the immunogen to multiply, said protein-wasting immunogen is P antigen from *P. anaerobius*, which method comprises:

A. Inoculating female birds, in or about to reach their egg laying age, with P antigen with *P. anaerobius*;

B. Allowing a period of time sufficient to permit the production of the bird and eggs laid by the birds of antibody to P antigen from *P.anaerobius*, said antibody in the eggs including IgY immunoglobulins in the yolks of the eggs and IgM and IgA immunoglobulins in the albumin of the eggs;

C. Harvesting the eggs laid by the birds;

D. Separating the entire contents of said harvested eggs from the egg shells;

E. Providing a dry feed carrier material; and

F. Coating said dry feed carrier material with the separated entire contents of said harvested eggs, said dry food carrier material coated with the entire contents of said eggs when administered to the food animals inhibiting the adherence of colony-forming immunogen in the digestive tract by binding the IgY immunoglobulins to the colony-forming immunogen, said binding of the IgY immunoglobulins to the colony-forming immunogen being assisted by the IgM and IgA immunoglobulins.

33. The microbial adherence inhibitor according to Claim 32 wherein: the dry feed carrier material is from a group of materials including soybean hulls, rice hulls, corn, cottonseed hulls, distilled dried grains and beet pulp.

34. A method for the production of a microbial adherence inhibitor for administration to food animals to inhibit the ability of the immunogen to adhere to the rumen or intestinal tracts of food animals to reduce the ability of the immunogen to multiply, said protein-wasting immunogen is CS antigen from *C. sticklandii* produced by the method of:

A. Inoculating female birds, in or about to reach their egg laying age, with CS antigen from *C. sticklandii*;

B. Allowing a period of time sufficient to permit the production in the bird and eggs laid by the birds of antibody to CS antigen from *C. sticklandii*, said antibody in the eggs including IgY immunoglobulins in the yolks of the eggs and IgM and IgA immunoglobulins in the albumin of the eggs;

C. Harvesting the eggs laid by the birds;

D. Separating the entire contents of said harvested eggs from the egg shells;

E. Providing a dry feed carrier material; and

F. Coating said dry feed carrier material with the separated entire contents of said harvested eggs, said dry food carrier material coated with the entire contents of said eggs when administered to the food animals inhibiting the adherence of colony-forming immunogen in the digestive tract by binding the IgY immunoglobulins to

the colony-forming immunogen, said binding of the IgY immunoglobulins to the colony-forming immunogen being assisted by the IgM and IgA immunoglobulins.

35. The microbial adherence inhibitor according to Claim 34 wherein: the dry feed carrier material is from a group of materials including soybean hulls, rice hulls, corn, cottonseed hulls, distilled dried grains and beet pulp.

36. A method for the production of a microbial adherence inhibitor for administration to food animals to inhibit the ability of the immunogen to adhere to the rumen or intestinal tracts of food animals to reduce the ability of the immunogen to multiply, said protein-wasting immunogen is CA antigen from *C. aminophilium* produced by the method of:

A. Inoculating female birds, in or about to reach their egg laying age, with CA antigen from *C. aminophilium*;

B. Allowing a period of time sufficient to permit the production in the bird and eggs laid by the birds of antibody to CA antigen from *C. aminophilium*, said antibody in the eggs including IgY immunoglobulins in the yolks of the eggs and IgM and IgA immunoglobulins in the albumin of the eggs;

C. Harvesting the eggs laid by the birds;

D. Separating the entire contents of said harvested eggs from the egg shells;

E. Providing a dry feed carrier material; and

F. Coating said dry feed carrier material with the separated entire contents of said harvested eggs, said dry food carrier material coated with the entire contents of said eggs when administered to the food animals inhibiting the adherence of

colony-forming immunogen in the digestive tract by binding the IgY immunoglobulins to the colony-forming immunogen, said binding of the IgY immunoglobulins to the colony-forming immunogen being assisted by the IgM and IgA immunoglobulins.

37. The microbial adherence inhibitor according to Claim 36 wherein: the dry feed carrier material is from a group of materials including soybean hulls, rice hulls, corn, cottonseed hulls, distilled dried grains and beet pulp.

38. A method for the production of a microbial adherence inhibitor for administration to a living being to inhibit the adherence of a colony forming immunogen in the digestive tract of the living being, said colony-forming immunogen is from the class consisting of *E. coli*, *Listeria*, *Salmonella* and *Campylobacter*, which method comprises:

A. Inoculating female chickens in or about to reach their egg laying age with the colony-forming immunogen;

B. Allowing a period of time sufficient to permit the production in the eggs of the chickens of antibody to the colony-forming immunogen, said antibody in the eggs including IgY immunoglobulins in the yolks of the eggs and IgM and IgA immunoglobulins in the albumin of the eggs;

C. Harvesting the eggs laid by the chickens;

D. Separating the entire contents of said harvested eggs from the egg shells; and

E. Providing a dry carrier material; and

F. Coating said dry carrier material with the separated entire contents of said harvested eggs, said dry carrier material coated with the entire contents of said eggs when administered to the living being inhibiting the adherence of colony-forming immunogen

in the digestive tract by binding the IgY immunoglobulins to the colony-forming immunogen, said binding of the IgY immunoglobulins to the colony-forming immunogen being assisted by the IgM and IgA immunoglobulins.

Oral passive immunization against experimental salmonellosis in mice using chicken egg yolk antibodies specific for *Salmonella enteritidis* and *S. typhimurium*

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The efficacy of chicken egg yolk homotypic antibodies specific for outer membrane proteins (OMP), lipopolysaccharide (LPS) or flagella (Fla) in controlling experimental salmonellosis in mice was investigated. Mice challenged orally with 2×10^9 c.f.u. of Salmonella enteritidis or 2×10^9 c.f.u. of S. typhimurium were orally treated with 0.2 ml anti-OMP, -LPS or -Fla yolk antibody three times a day for three consecutive days. In mice challenged with S. enteritidis, antibody treatment resulted in a survival rate of 80%, 47% and 60% using OMP, LPS or Fla specific antibodies respectively, in contrast to only 20% in control mice. In the S. typhimurium trial, survival rate was 40%, 30% and 20% using OMP, LPS or Fla specific antibodies respectively in contrast to 0% in control mice. In vitro adhesion of S. enteritidis and S. typhimurium to HeLa cells was significantly reduced by anti-OMP, -LPS, and -Fla homotypic antibodies. Results suggest that egg yolk antibodies specific for Salmonella OMP, LPS, and Fla may protect mice from experimental salmonellosis when passively administered orally. Of these antibodies, anti-OMP exhibited the highest level of protection in vivo and in vitro. © 1998 Elsevier Science Ltd. All rights reserved

Keywords: egg yolk antibody; passive immunization; *Salmonella*

Salmonella enteritidis and *S. typhimurium* are a non-host restricted serotype which can cause disease syndromes like gastroenteritis and systemic infections in a wide range of animal species. In the distal ileum, salmonellae adhere to and pass through intestinal epithelial cells, primarily the M cells of the follicle-associated epithelium¹⁻³.

Many approaches have been employed to investigate the mechanisms of pathogenicity of *Salmonella*⁴⁻⁸. Genetic and molecular studies on plasmid and genes encoding for virulence determinants have been reported⁹⁻¹¹. *In vitro* studies of bacterial adhesion and invasion have been facilitated by tissue culture assays on isolated intestinal epithelial cells and epithelial cell monolayers¹²⁻¹⁶.

Salmonella has a variety of surface components which are virulence-related. Among them, OMP also known as porins, play a role as pathogenicity determi-

nants¹⁷. OMP are exposed on the surface of the bacterial cell and they can serve as phage receptors and react with antibodies¹⁸. Numerous studies have elucidated their biological functions and immunogenic properties^{19,20}. With their physical and biological characteristics, OMP have been used successfully as vaccine antigens and a number of workers have proven that they are good immunogens and are protective in both active and passive immunization studies involving mice with sera as the source of protective antibodies^{18,21-24}. OMP and LPS are required to elicit an effective cellular immune response and antibodies with immunoprotective potential^{25,26}. Monoclonal antibodies that presumably recognized epitopes present in porin-LPS complexes were also protective against endotoxemia and mouse typhoid²⁷.

Motility seems to increase the probability that bacteria will reach suitable sites for invasion^{28,29}. Active flagella facilitate movement towards host cells *in vitro*. The absence of flagella during infection does not reduce the ability of salmonellae to enter cultured cells or initiate disease by penetration of the Peyer's patches, and the presence of inactive flagella seems to

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attenuate the ability of the organism to interact with the host³⁰.

A correlation between the presence of fimbriae and bacterial virulence has been demonstrated in salmonellosis with a range of fimbrial types³¹. Conversely, decreased virulence was due either to loss of fimbriation or to the inhibitory action of anti-fimbrial antibodies on bacterial adhesion³². In another study, bacterial virulence was reduced by passive immunization with chicken egg yolk anti-fimbrial antibodies³³.

The aim of the present study was to determine the efficacy of chicken egg yolk derived antibodies specific for salmonellae OMP, LPS, and Fla in the control of experimental salmonellosis in mice and in adhesion inhibition *in vitro*. In the *in vivo* trial, the effect of passive immunization of anti-OMP, -LPS, and -Fla antibodies on the excretion pattern and persistence of *S. enteritidis* and *S. typhimurium* in infected mice was characterized.

MATERIALS AND METHODS

Mice

A total of 100 BALB/c SPF mice (Charles River Japan Inc., Kanagawa, Japan) 4 weeks old were randomly distributed into two trials.

Bacterial strains

S. enteritidis strain GIFU-3161 (ATCC-13076) and *S. typhimurium* strain GIFU-4892 (ATCC-13311) were obtained from Dr T. Ezaki, Gifu University, School of Medicine, Gifu City, Japan. These isolates were grown in Luria broth at 37°C for 18 h. For inactivation, whole bacteria were suspended in 0.5% formalin for 18 h at 37°C in PBS.

Preparation of outer membrane proteins

OMP was prepared using the method of Nurminen³⁴ and Schnaitman³⁵ with modifications. Bacteria were suspended in 0.01 M HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid) buffer and homogenized. After centrifugation at 12000 g at 4°C, the bacterial cells were resuspended in 0.01 M HEPES and sonicated. Centrifugation was repeated and supernatant was collected and subjected to ultracentrifugation at 200000 g for 45 min at 4°C. Bacterial pellet was treated with 2% Triton-X100 in 0.01 M HEPES and ultracentrifuged. The procedure was repeated with 2% Triton-X100 and 5 mM EDTA in 0.01 M HEPES, and ultracentrifuged. The sediment was resuspended in PBS and dialysed against the same buffer. The protein content of the sample was determined by a Bio-Rad protein assay system (Bio-Rad Laboratories, Calif., USA). The purity of each OMP preparation was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 5–20% gradient acrylamide gels (Atto Co., Tokyo, Japan) with prestained standards (Bio-Rad).

Preparation of lipopolysaccharide

The conventional phenol extraction procedure was used³⁶. Briefly, bacteria cells were suspended in distilled water. An equal volume of 90% (W/V) phenol

preheated to 68°C was added, and the resultant mixture was stirred for 20 min and centrifuged. Aqueous phases were centrifuged, dialysed against distilled water, centrifuged again and lyophilized.

Crude LPS preparations were suspended in 10 mM Tris-HCl buffer (pH 7.5) with NaCl concentrations 10 mM. The LPS was applied onto a ion-exchange chromatographic column packed with Q Sepharose First flow (Pharmacia Biotech, Uppsala, Sweden). For fractionation, 10 mM Tris-HCl buffer with increasing NaCl concentrations (0.2, 0.3, 0.4, and 1.0 M) was used as eluent. Fractions were collected and protein was monitored spectrophotometrically at 280 nm. The solution was dialysed against PBS and the LPS preparation was analysed by SDS-PAGE.

Preparation of flagella

The conventional homogenized extraction procedure was used. Bacteria were suspended in PBS and homogenized for 2 min. After centrifugation to remove the cells, the supernatant was collected and subjected to ultracentrifugation at 40000 g for 4 h. The pellet was treated in 0.1 M Tris-HCl buffer (pH 8.0). The flagella was applied onto an ion-exchange chromatographic column packed with DEAE-Sephadex A-50 (Pharmacia). For fractionation, 0.1 M Tris-HCl buffer (pH 8.0) with increasing NaCl concentrations (0.3, 0.5, 0.7, and 1.0 M) was used as eluent. Fractions were collected and protein was monitored spectrophotometrically at 280 nm. The solution was dialysed against PBS and the protein content and purity of the pooled sample was determined. Purity of the flagellar preparation was analysed by SDS-PAGE and further confirmed by transmission electron microscope (H-300, Hitachi, Tokyo, Japan) examination of negatively stained samples.

Immunization of chickens

Five-month-old White leghorn chickens (strain Hyline W36; GHEN Corporation, Gifu, Japan) were immunized with OMP, LPS, and Fla extracted from *S. enteritidis* and *S. typhimurium* for the induction OMP, LPS, and Fla specific antibodies in egg yolk. The antigen consisting of 0.5 mg of either OMP, LPS or Fla was emulsified in emulsion oil mixed with 5% sorbitan monooleate (Maine Biological Laboratories, Maine, USA) as adjuvant and injected intramuscularly into the breast muscles of chickens. Eight weeks after the first injection, chickens received booster injections of the same dosage by the same route of administration and this was repeated two weeks later. Eggs were collected daily starting from the second week after the last booster until enough eggs were pooled and processed.

Isolation of antibodies from chicken egg yolk

The egg yolk Immunoglobulin G (IgG) was prepared using the procedure in an earlier study³⁷. The separated yolks were diluted with 8 volumes of distilled water. An aqueous suspension of 5% hydroxypropylmethylcellulose phthalate (Shinetsu Chemical Inc., Tokyo, Japan) in 80% ethyl alcohol was added to the diluted egg yolk gradually, and the mixture was centrifuged at 12000 g for 20 min. The supernatant which contained the IgG fraction was purified by affinity chromatography using

Avid AL gel (BioProbe International Inc., Tustin, CA, USA). Elution of the sample was accomplished by 0.05 M sodium acetate buffer (pH 3.0). The solution was dialysed against PBS, and analysed for antibody titer and purity by SDS-PAGE.

Titration of antibodies

The antibody titer against the challenge-exposure strain was determined by a microtitration plate agglutination method. Inactivated whole bacteria of *S. enteritidis* and *S. typhimurium* were used as antigen in the agglutination test. Samples of bacterial suspension were added to PBS to a desired optical density (0.42 at 620 nm). The adjusted bacterial suspension was used as antigen; 0.05 ml was added to equal volumes of twofold serial dilutions of an antibody solution in PBS. After 2 h incubation at 37°C and overnight incubation at 4°C, the titer was read as the highest dilution of antibody showing agglutination.

Challenge exposure of mice to *Salmonella* and oral administration of antibodies

The succeeding procedures in the challenge exposure of mice were performed as described elsewhere³¹. Trial 1: Four groups with 15 mice per group was inoculated orally with 0.2 ml of *S. enteritidis* suspension (1×10^{10} c.f.u. ml⁻¹). Trial 2: Four groups with ten mice per group were inoculated orally with 0.2 ml of *S. typhimurium* (1×10^8 c.f.u. ml⁻¹). All mice in each trial were given 0.2 ml of the challenge infectious dose as specified above and about 30 min later, 0.2 ml antibody solution consisting of anti-OMP, anti-LPS, anti-Fla and control antibody from sham immunized chickens were given to groups 1, 2, 3, and 4 respectively. The antibody solution was given daily to each mouse three times a day for three consecutive days. The bacterial inoculum and antibodies were administered by gastric intubation using a sterilized blunt needle attached to a 1 ml tuberculin syringe. Mice were observed daily for two weeks for clinical signs of infection and survival (%) of mice was calculated.

Enumeration of *Salmonella* in organ homogenates

At the end of the trial, surviving mice were killed by quick cervical dislocation and spleen, liver, and cecum were collected. The organ samples were homogenized in PBS, subcultured in protein water (Difco, Detroit, Mich., USA), enriched in Selenite broth (Nissui Pharmaceutical Co., Tokyo, Japan) incubated, and then cultured on desoxycholate-hydrogen sulfide-lactose agar (DHL agar: Nissui) and confirmed by positive O4 and O9 antiserum agglutination test (Denka Seiken Co., Tokyo, Japan).

***In vitro* adherence of *Salmonella* to HeLa cell and inhibition by antibodies**

The succeeding procedures in the adhesion assay were performed as described elsewhere¹⁶ with modification. Bacteria were suspended at a concentration of approximately 1×10^7 c.f.u. ml⁻¹ in Eagle's minimal essential medium without kanamycin (MEM No.3,

Nissui). HeLa S3 cell monolayers (ATCC CCL-2.2; Flow Laboratories, McLean, Va., USA) were prepared by placing 10^5 cells in MEM medium into each chamber of Lab-Tek tissue culture chamber/slides (Lab-Tek, #4804, Miles Lab., Naperville, Ill., USA). After a period of incubation, the medium was aspirated from each chamber and 1 ml suspension of *Salmonella* or *Salmonella*+antibody was added. Inhibition of bacterial attachment to HeLa cells was done by pre-incubating the mixture of 1 ml of bacteria with 1 ml of egg yolk antibody solution in MEM (1:10 dilution of original purified IgG sample) for 15 min and followed by twofold serial dilution of the mixture. One millilitre of the serially diluted mixture was added onto Lab-Tek chambers with HeLa cell as described above followed by incubation at 37°C for 30 min. The slide chambers were then washed thrice with warm PBS, fixed with cold acetone, and stained with Giemsa solution. The number of bacteria attached to 20 HeLa cells was determined and the average number of bacterial cells attached to each HeLa cell was calculated. The minimum adhesion inhibitory concentration titer was determined by the formula: antibody titer/dilution of antibody solution with significant ($p < 0.01$) adhesion-inhibition assay.

Statistical analysis

The statistical significance of differences in survival rates between the treated and control groups was assessed by using the Fisher's exact test, and the mean differences in bacterial adhesion *in vitro* between the control and pre-incubated groups was assessed by the Student's *t* test.

RESULTS

Antibody titer to *salmonella*

The immunological specificity of egg yolk antibody was assessed by a microtitration plate agglutination method. The titer of the OMP, LPS, and Fla specific antibody solution for *S. enteritidis* were 256, 128, and 1024 respectively (Table 1). Those for the OMP, LPS, and Fla antibody solution for *S. typhimurium* were 256, 128, and 2,048 respectively (Table 2). The titer of egg yolks of non-immunized hens was <2 against the inactivated whole bacteria antigens used in the assay.

Clinical evaluation of mice after challenge exposure with *Salmonella* and passive immunization with antibody

Trial 1. The clinical responses of mice after *S. enteritidis* challenge and subsequent treatment with antibodies are shown in Figure 1. Mice in Group 1 (OMP antibody: titre 1:256) showed a survival rate of 80%, from *S. enteritidis* infection. Mice in Group 2 (LPS antibody: titer 1:128) had a 47% survival rate. Mice in Group 3 (Fla antibody: titer 1:1024) had 60%, whereas control mice in Group 4 administered non-immune egg yolk antibodies had the lowest survival rate at 20%. The difference in the survival rates between the OMP antibody group and the control group was statistically significant ($p < 0.05$). The

Table 1 Effect of egg yolk antibody on *in vitro* adhesion of *S. enteritidis* to HeLa cells

Antibody titer		Mean no. of adherent <i>Salmonella</i> cells at different antibody dilution					Minimum inhibitory titer ^a
		× 10	× 20	× 40	× 80	× 160	
OMP	256	6+6 ^{b**}	10+4 ^{**}	21+7 ^{**}	23+14 ^{**}	36+12	3.2
LPS	128	12+8 ^{**}	17+9 ^{**}	25+8 ^{**}	33+12	39+11	3.2
Fla	1024	23+17 ^{**}	24+8 ^{**}	33+12	43+15	39+13	51.2
Control	<2	42+12					

^aMinimum adhesion inhibitory concentration = Antibody titer/Dilution of antibody solution with significant adhesion inhibition^bNumber of bacteria attached per HeLa cell+Standard deviation^{**}*p* < 0.01 relative to the control (Student's *t* test)

susceptible mice manifested the following clinical signs after infection: lethargy, anorexia, and death. All mice in the control group which survived *S. enteritidis* infection harboured the organism in the liver and spleen regardless of whether excretion in the faeces had ceased or not at the end of experiment. On the other hand, 50% in the OMP group, 86% in the LPS group and 56% in the Fla group were positive for *S. enteritidis* in the liver and spleen organism.

Trial 2. The clinical responses of mice after *S. typhimurium* challenge and subsequent treatment with antibodies are shown in Figure 2. Mice in Group 1 (OMP antibody: titre 1:256) showed a 40% survival rate. Mice in Group 2 (LPS antibody: titre 1:128) had a 30% survival rate. Mice in Group 3 (Fla antibody: titre 1:2048) had a 20% survival rate whereas control mice in Group 4 administered non-immune egg yolk antibodies all died. The difference in the survival rates between the OMP antibody group and the control group was statistically significant (*p* < 0.05). The susceptible mice manifested the following clinical signs after infection: lethargy, anorexia, and death. On the other hand, 25% in the OMP group, 100% in the LPS group, and 0% in the Fla group were positive for *S. typhimurium* in the liver and spleen organism.

In vitro adherence-inhibition assay

Salmonella adhered to HeLa cells in varying degrees. The average number of adherent bacteria per HeLa cell was 42 for both *S. enteritidis* (Table 1) and *S. typhimurium* (Table 2). The attachment of bacteria to HeLa cells was reduced by homologous anti-OMP, -LPS, and -Fla antibodies. The minimum antibody titer that significantly inhibited adherence compared to control was 3.2 for OMP, 3.2 for LPS and 51.2 for Fla in the case of *S. enteritidis* homotypic antibodies. For antibodies

homologous to *S. typhimurium*, the figures were the same except for LPS antibody which was 6.4.

DISCUSSION

In this report, we examined the effect of oral passive immunization with egg yolk antibodies with specificities against OMP, LPS, and Fla of either *S. enteritidis* and *S. typhimurium* in controlled experimental salmonellosis in mice. The present method of adoptive antibody transfer can serve as a tool to elucidate the importance and role of OMP, LPS, and Fla in the virulence of salmonellae in mice. Particularly, the OMP or porins play a role as pathogenicity determinants. They are exposed on the surface of the bacteria and can interact with cellular and humoral systems^{17,38}. The passive transfer of anti-OMP antibodies may therefore influence colonization by *S. enteritidis* and *S. typhimurium* by binding with these surface proteins. Ishibashi *et al.*²¹ demonstrated in a mouse model that *Salmonella* OMP induces protection against challenge with *S. typhi*. The OMP produced in that study had 4% LPS contamination and was purified according to the method of Schnaitman *et al.*³⁵. Using the anti-OMP antibodies pre-absorbed with LPS, they were able to induce protection in mice.

It appears that aside from microbial characteristics, there are certain host factors which affect the ability of *Salmonella* serotype to survive, multiply, and produce systemic disease. Host factors in adult animals include increased resistance to salmonellosis, attributed to greater immunological maturity of the reticulo-endothelial system³⁹ and microbiological maturity of the gut⁴⁰. The pattern of excretion of *Salmonella* in the faeces of mice between the antibody and control groups was roughly similar within 24 h of oral inoculation of the organisms (data not shown). High viable bacterial counts were detected on day 1 post-challenge and decreased gradually within 72 h. It was during this

Table 2 Effect of egg yolk antibody on *in vitro* adhesion of *S. typhimurium* to HeLa cells

Antibody titer		Mean no. of adherent <i>Salmonella</i> cells at different antibody dilution					Minimum inhibitory titer ^a
		× 10	× 20	× 40	× 80	× 160	
OMP	256	3+4 ^{b**}	9+13 ^{**}	15+9 ^{**}	26+10 ^{**}	36+10	3.2
LPS	128	15+8 ^{**}	31+12 ^{**}	38+12	42+11	46+12	6.4
Fla	2048	12+9 ^{**}	15+7 ^{**}	27+7 ^{**}	39+10	43+15	51.2
Control	<2	42+15					

^a Same as in Table 1^b Number of bacteria attached per HeLa cell+Standard deviation^{**}*p* < 0.01 relative to the control (Student's *t* test)

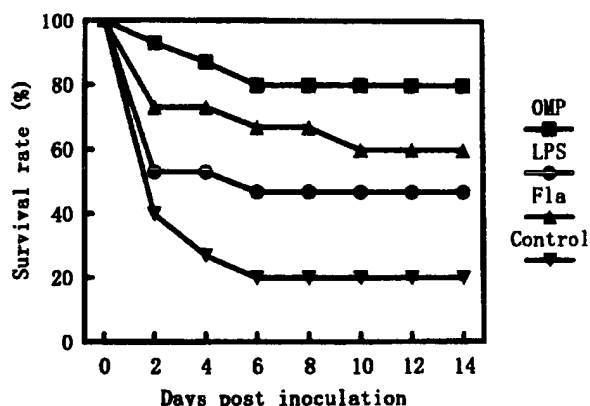


Figure 1 Protection of mice orally administered with egg-yolk antibody to *S. enteritidis*

peak period that the most mortalities were observed in mice (Figures 1 and 2). The control mice were continuously shedding *Salmonella* in their faeces, with gradually decreasing viable bacterial counts but evident until two weeks post-infection. Post-mortem bacteriological examination showed various degrees of colonization by *Salmonella* in the liver, spleen, and cecum (data not shown). This suggested a rapid proliferative growth in different organs leading to a systemic disease.

Excretion of *Salmonella* via faeces was eliminated earlier among mice treated with anti-OMP antibodies compared to controls. In the antibody treated group, intermittent excretion of *Salmonella* was generally observed, but viable counts were lower compared to those of the untreated control group.

Salmonella adhered to HeLa cells in varying degrees. The attachment of bacteria to HeLa cells was reduced by homologous anti-OMP, -LPS, and -Fla antibodies. The minimum adhesion inhibitory titers of anti-OMP, -LPS and -Fla antibodies that significantly ($p < 0.01$) inhibited adhesion compared to controls was similar for both *S. enteritidis* and *S. typhimurium* (Tables 1 and 2). From the data, it appears that anti-OMP had a slight advantage over LPS or Fla specific antibodies.

The difference in survival rates after antibody treatment between *S. enteritidis*- and *S. typhimurium*-infected mice despite similar *in vitro* protection rates by

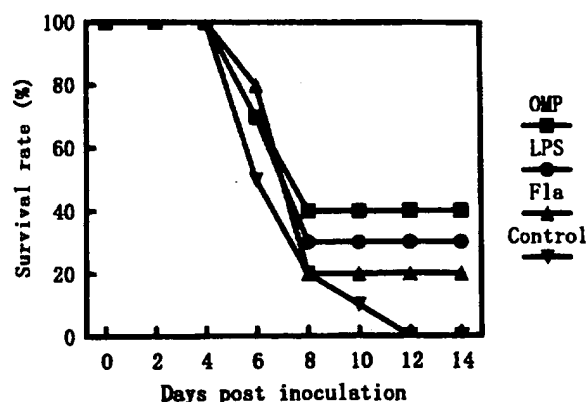


Figure 2 Protection of mice orally administered with egg-yolk antibody of *S. typhimurium*

the antibodies may be attributed to a combination of several factors. *In vitro*, efficacy was judged according to adhesion inhibition only whereas *in vivo*, similar levels of adhesion inhibition may have different colonization outcome due to other factors such as bacterial pathogenicity, challenge dose and host susceptibility.

The efficacy of anti-OMP egg yolk antibodies can be more appreciated in view of the protection it conferred on mice which appeared to be more susceptible to salmonellosis. The precise mechanisms by which anti-OMP antibodies protect against *Salmonella* invasion of the host is not clearly known. On the basis of the present knowledge on outer membrane proteins of gram-negative bacteria and their biological functions, studies on adhesion to and invasion of the mucosa by *Salmonella*, as well as experimental results from our protection trials, we hypothesize that OMP, since they are exposed on the surface of the bacterial cell, can easily be recognized by antibodies. The subsequent binding may lead to impairment of the biological functions of OMP by disrupting the interaction and presentation of other cell surface components which may also play a role in virulence. The end result is a reduced invasiveness of *Salmonella* with loss of ability to colonize the intestinal tract in numbers sufficient to cause a fatal disease. The use of passively administered egg antibodies may be extended to other species in the form of oral egg antibody preparations.

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